Detection and Differentiation of Parasitoids (Hymenoptera: Aphidiidae and Aphelinidae) of the Brown Citrus Aphid (Homoptera: Aphididae): Species-Specific Polymerase Chain Reaction Amplification of 18S rDNA

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ABSTRACT The brown citrus aphid, *Toxoptera citricida* (Kirkaldy), is an important pest of Florida citriculture because it causes feeding damage to citrus and vectors citrus tristeza virus. Parasitoids recovered from brown citrus aphids in Florida include *Lysiphlebus testaceipes* (Cresson), *Lipolexis scutellaris* Mackauer, and *Aphelinus gossypii* Timberlake. Monitoring the levels of parasitism caused by each species is difficult because the parasitoids must be reared out or dissected from the aphid hosts. A simple and quick molecular approach was developed to detect and distinguish these parasitoids developing within the host aphid. Total genomic DNA was extracted from the brown citrus aphid and each of the three parasitoids and the 18S rRNA gene of each species was amplified by polymerase chain reaction (PCR). The PCR products were sequenced to obtain complete gene sequences for each species. The variable regions V2 of the genes were used to design species-specific primers for detecting and differentiating the three parasitoids. The species-specific PCR amplifications discriminated the parasitoid DNAs from each other and from the host DNA. Detection of *L. testaceipes* DNA within the host aphid was possible in 8% of samples during the first 2 h after parasitoid oviposition; in 66% of samples after 24 h; in 94% of samples after 48 h; and in 100% of samples after 72 h. The PCR approach described in this study provides earlier and more precise detection of parasitism and determination of species than rearing or dissection methods.

KEY WORDS *Toxoptera citricida*, *Lysiphlebus testaceipes*, *Lipolexis scutellaris*, *Aphelinus gossypii*, 18S rDNA

THE BROWN CITRUS APHID, *Toxoptera citricida* (Kirkaldy), is a harmful pest of citrus because it damages the young growing shoots of infested plants and efficiently vectors certain virulent strains of citrus tristeza virus (Yokomi et al. 1994). This exotic pest was first detected in southern Florida in 1995 (Halbert and Brown 1996) and is now distributed throughout the citrus-producing regions of the state (Liu and Tsai 2002). Integrated control strategies for brown citrus aphid are dependent on pesticides, cultural practices, and the use of native and imported natural enemies. Management of brown citrus aphid populations through conservation and importation of biological control agents should assist in reducing the spread of citrus tristeza virus.

Yokomi and Tang (1996) previously considered the brown citrus aphid a poor host for the native parasitoid *Lysiphlebus testaceipes* (Cresson). They found emergence rates of parasitoid adults from brown citrus aphid mummies were <5% in Puerto Rico. Michaud (1998) reported that *L. testaceipes* often parasitized brown citrus aphid in Florida but aphid mummies with emergence holes were difficult to find. Subsequently, *L. testaceipes* was collected from brown citrus aphid in Orlando, FL, and has been reared on this host at the U.S. Horticultural Research Laboratory since 1999 (Tang et al. 2002). The emergence rate of adult parasitoids in the colony exceeds 80%, and this laboratory-selected strain has been released in several locations, including Brevard, Indian River, Orange, and St. Lucie counties, Florida (A.A.W., unpublished data). *L. testaceipes* is known for its generalist behavior with regard to host aphid species (Starý 1993, Gonzales et al. 2002) and seems to be adapting to the exotic brown citrus aphid.

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The imported parasitoids *Aphelinus gossypii* Timberlake and *Lipolexis scutellaris* Mackauer have been introduced to enhance biological control of brown citrus aphids in Florida (Denmark and Porter 1973, Evans and Stange 1997, Hoy and Nguyen 2000). Other parasitoids have been imported and released over time, including *Lysiphlebia japonica* (Ashmead) and *Lysiphlebia mirzai* Shuja-Uddin; however, these apparently have not established (Evans and Stange 1997, Liu and Tsai 2002). *L. testaceipes,* by far, is the most common primary parasitoid of brown citrus aphids in Florida; *A. gossypii* is established and has been collected from brown citrus aphids, but it is more often reared from other aphid species; and although *L. scutellaris* is rarely recovered from brown citrus aphid, its potential may not have been realized because it was only recently introduced (Evans and Stange 1997; G.A.E., unpublished data).

Monitoring the levels of parasitism caused by multiple parasitoid species attacking the same host aphid is difficult, time-consuming, and often inaccurate because the parasitoids must be reared to adulthood or the larvae dissected from the aphid hosts in the laboratory. Weathersbee and Hardee (1994) reared field-collected aphids from cotton in the laboratory for 6 d and used mummy formation to estimate seasonal parasitism by *L. testaceipes.* Additional rearing through adult parasitoid emergence was required to confirm the presence of *L. testaceipes.* These estimates did not account for unknown mortality that may have occurred during rearing. Using host specimens of Curculionidae and Miridae as experimental models, Day (1994) demonstrated that parasitism measured by dissection was higher and more accurate than that determined by rearing. Differences in the two methods were attributed to mortality that occurred during the rearing process. He indicated that supplemental rearing was often required with dissection for identification of parasite species. An alternative method is needed to detect and identify brown citrus aphid parasitoids, because dissection and identification of early instars are particularly difficult. In this experiment, a simple and quick molecular approach was developed to detect and identify brown citrus aphid parasitoids during early development within the aphid hosts.

Materials and Methods

Insect Sources and Rearing. Laboratory colonies of the brown citrus aphid and *L. testaceipes* were established from field-collected specimens and maintained on young foliage of potted sour orange, *Citrus aurantium* L., or 'Carrizo' citrange, *Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf., seedlings in screened cages measuring 45 by 45 by 70 cm. A colony of *A. gossypii* originally imported from China was maintained in the same manner until August 2002. Specimens of *L. scutellaris* were obtained from the Florida Department of Agriculture and Consumer Services, Division of Plant Industry. Representative samples of all four species were stored at −80°C and in 95% ethanol at −10°C for genomic analyses.

Table 1. Primers used to PCR amplify and sequence, whole and partial, 18S rDNA products from *T. citricida*, *A. gossypii*, *L. scutellaris*, and *L. testaceipes* genomic DNAs

Primer name[a]	Primer sequence	Tm[b]
18SFrontF*	CTGGTTGATCCTGCCAGTAGT	57.65
18SFrontR*	GGTTAGAACTAGGGCGGTATC	56.37
18SBackF*	GATACCGCCCTAGTTCTAACY	55.11
18SBackR*	TCCTTCCGCAGGTTCACC	59.58
18SFrontI1F	GTCTGCCTTATCAACTGTCGA	56.22
18SFrontI1R	TCGACAGTTGATAAGGCAGAC	56.22
18SFront12F	TTACTTTGAACAAATTAGAGTGCT	54.66
18SFront12R	AGCACTCTAATTTGTTCAAAGTAA	54.66
18SBackI1F	AGCTCTTTCTTGATTCGGTGG	59.70
18SBackI1R	CCACCGAATCAAGAAAGAGCT	59.70
18SBackI2F	TTAGATGTTCTGGGCCGC	57.51
18SBackI2R	GCGGCCCAGAACATCTAA	57.51
18SFrontR-AG	AAACGGTGGACGAGCAAG	57.35
18SFrontR-LS	ATTAACGGTGGACAAATAAACT	54.39
18SFrontR-LT	ATTAACGATGGATATATAACCGA	54.82

[a] Primers followed by an asterisk were reported by Campbell et al. (1994). Abbreviations in primer names: F, forward; R, reverse; I, internal; AG, *A. gossypii;* LS, *L. scutellaris;* and LT, *L. testaceipes.* "Front" and "Back" refer to 5′ and 3′ sections of the 18S rRNA gene.
[b] Melting temperatures of primers (°C).

Isolation of Genomic DNA. Total genomic DNA was extracted from single individuals of brown citrus aphid and each of the parasitoid species by using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). Genomic DNA was isolated following the manufacturer's protocol for animal tissue with modifications described previously (Weathersbee et al. 2003) to account for the small size of the insects. Briefly, an insect was ground in 300 μl of chilled EDTA/Nuclei Lysis Solution buffer (60 μl of 0.5 M EDTA, 250 μl of Nuclei Lysis Solution); then, 3 μl of 20 mg/ml Proteinase K was added and the sample was incubated overnight at 55°C. The manufacturer's protocol was used without changes for the remaining procedures apart from resuspending the purified DNA in only 30 μl of rehydration solution.

Polymerase Chain Reaction (PCR) and Sequencing of 18S rDNA. Total genomic DNA was isolated from each of the four insect species and the entire 18S rRNA gene was amplified by PCR with the primers 18SFrontF and 18SBackR previously reported by Campbell et al. (1994) (Table 1). A PCR Core kit (QIAGEN, Valencia, CA) was used to prepare the reaction mixtures (50 μl) containing 1× PCR buffer solution, 1× Q solution, 0.2 mM of each dNTP, 0.2 μM of each primer, 1.25 U of *Taq*DNA polymerase, and 50 ng of template. Amplifications were performed using a PTC-100 thermocycler (MJ Research, Watertown, MA) with PCR conditions as follows: 2 min at 98°C; then 35 cycles of 1 min at 96°C, 1 min at 56°C, and 1 min at 72°C; and a final extension step of 7 min at 72°C.

The amplified products were purified with a QIAquick PCR purification kit (QIAGEN), and were sequenced directly in both directions on an ABI PRISM 3700 DNA Analyzer by using Big Dye Version 3 chemistry (Applied Biosystems, Foster City, CA). Primers

Fig. 1. Clustal W alignment of partial sequences from *L. scutellaris*, *L. testaceipes*, *A. gossypii*, and *T. citricida* 18S rRNA genes. The locations of the priming sites for the conserved forward primer 18SFrontF and the conserved reverse primer 18SFrontI1R are in bold case. The species-specific reverse primers, 18SFrontR-LS, 18SFrontR-LT, and 18SFrontR-AG, are in bold case and shaded boxes.

used in the sequencing reactions are shown in Table 1. TraceTuner (Paracel, Pasadena, CA) software was used to analyze nucleotide base calls for data quality. Contiguous sequences were generated using Sequencher (Gene Codes, Ann Arbor, MI) software, and a consensus was obtained from replicates of at least three individuals per species. Sequences were aligned using the default parameters for CLUSTAL W (Thompson et al. 1994). The complete 18S rRNA gene sequences for all four species were deposited in the GenBank database under the consecutive accession numbers AY216697–AY216700.

Analyses of Species-Specific Primers. Assessment of the aligned 18S rDNA sequences at variable region V2 revealed a section, corresponding to nucleotide positions 268–294 of *T. citricida*, that could be used to discriminate the parasitoid DNAs from one another and from the host DNA (Fig. 1). Species-specific reverse primers 18SFrontR-LT, 18SFrontR-LS, and 18SFrontR-AG were designed for this section and used with the conserved forward primer 18SFrontF in a PCR to assess the capacity of each primer set to specifically amplify the parasitoid DNAs (Table 1). The species-specific reverse primers also were used with the conserved forward primer in a PCR containing brown citrus aphid DNA to determine whether the host DNA was amplified. The conserved forward primer and the conserved internal reverse primer 18SFrontI1R were used to amplify brown citrus aphid DNA in a positive control (Table 1). Combinations of the conserved forward primer and each species-specific reverse primer were also used in PCRs containing mixtures of DNAs from the brown citrus aphid and each of the parasitoids. These reactions were performed in triplicate by using discrete mixtures of host and parasitoid DNAs. The PCR conditions were as described previously and the PCR products were separated on a 0.8% agarose gel.

Temporal Dependancy of Parasitoid Detection by PCR. Parasitoid detection by species-specific PCR was evaluated using third instars of the brown citrus aphid parasitized by newly emerged female *L. testaceipes* that were acclimated to aphids for ≤24 h. *L. testaceipes* was selected for this experiment because a colony of the parasitoid is maintained on brown citrus aphid at the U.S. Horticultural Research Laboratory, Fort Pierce, FL. The ability to detect the parasitoid by PCR was assessed at 0, 24, 48, and 72 h after a female wasp had oviposited in the aphid nymphs. Groups of 20–30 aphids on a sour orange citrus leaf were exposed to four to six parasitoids in a petri dish chamber (9 cm diameter by 2 cm in depth). Oviposition behavior was observed under a dissecting microscope to ascertain that only parasitized aphids were used in the experiment. For the 0-h assessment, each of 10 parasitized aphids was placed singly in a 1.5-ml centrifuge tube and frozen at −80°C within 2 h of observing the oviposition. For the 24-, 48-, and 72-h assessments, groups of 10 parasitized aphids were placed on flushing foliage of small citrus seedlings, and the parasitoids were allowed to develop for the appropriate interval. The aphids were then removed and each was frozen singly in a 1.5-ml centrifuge tube at −80°C. Five replicates, each containing 10 parasitized aphids, were assessed for each interval.

Genomic DNAs derived from each of the previously frozen, parasitized aphids were used to amplify 18S rDNA fragments by PCR by using the primers 18SFrontF and 18SFrontR-LT. The PCR conditions were as described previously, and the DNA fragments were separated on a 0.8% agarose gel. The percentage of positive parasitoid detections was recorded and analyzed. Linear regression was used to fit a quadratic model to the data to study the relationship between percentage of parasitoid infections detectable by PCR and time from parasitoid oviposition within the host aphid (SAS Institute 1999).

Results and Discussion

18S rDNA Sequences. The 18S rRNA genes were amplified by PCR as full-length products from the three parasitoid species and from the host aphid. Sequencing of the complete genes from at least three individuals of each species confirmed that the gene sequences were identical among individuals within each insect species. The CLUSTAL W (Thompson et al. 1994) alignment of the sequences from all four species indicated there was considerable interspecific heterogeneity among the sequences occurring in variable regions V2, V4, V7, and V9 of the genes. These variable expansion regions correspond to helices E10, E21, 41, and 47 of the 18S rRNA of the pea aphid, *Acyrthosiphon pisum* (Harris) (Kwon et al. 1991), and are useful in differentiating taxa based on interspecific genetic divergence (Campbell et al. 1994, 1995; Chong et al. 1999). The conserved 18S rRNA gene was used in this experiment instead of another less conserved ribosomal gene to avoid the concern raised by Scheffer et al. (2001) that recent divergence, particularly at subspecies levels, could lead to misidentification in some populations. Conversely, differentiation of insect populations below the species level requires a less conserved approach such as comparison of internal transcribed spacer two (ITS2) DNA sequences (Alvarez and Hoy 2002).

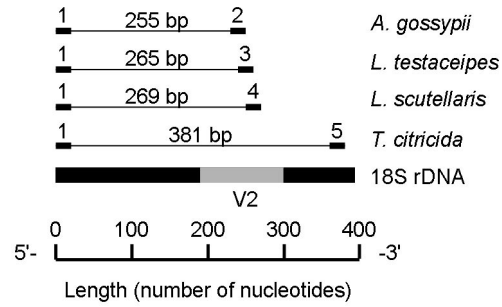

Fig. 2. Diagram of the relative size and location of the variable region (V2) within the first 400 nucleotides of the conserved 18S rRNA gene. Primers: 1, 18SFrontF; 2, 18SFrontR-AG; 3, 18SFrontR-LT; 4, 18SFrontR-LS; and 5, 18SFrontI1R. The primer annealing sites and sizes of the PCR products are shown for each insect species.

The length of each sequence was 2,480, 2,076, 1,932, and 1,905 base pairs (bp) for *T. citricida*, *L. scutellaris*, *L. testaceipes*, and *A. gossypii*, respectively. The length of the sequences determined for the brown citrus aphid and its parasitoids were similar to those reported for other aphid (Kwon et al. 1991) and wasp (Chalwatzis et al. 1995) species. The *T. citricida* sequence was considerably longer than those of the parasitoids due to nucleotide insertions particularly within the V4 and V7 expansion regions of the gene. The 18S rDNA sequence for *T. citricida* apparently is the longest known for an aphid species to date. The sequence length reported for the pea aphid was 2,469 bp (Kwon et al. 1991). Large expansion regions have been noted in the 18S rDNAs of other insects and particularly among the sternorrhynchans (Campbell et al. 1994, Chalwatzis et al. 1995).

The GC compositions were 60.1, 50.1, 46.8, and 42.5% for *T. citricida*, *A. gossypii*, *L. testaceipes*, and *L. scutellaris*, respectively. The total GC content of the brown citrus aphid sequence was higher than the reported average for insects (54.4%), whereas those for the parasitoids were lower than the average (Chong et al. 1999). These results concur with their finding of higher GC contents among unusually expanded 18S rRNA genes.

Parasitoid Detection and Differentiation. The annealing sites for the species-specific reverse primers were located near the 3′ end of the V2 variable region (Fig. 2). Each of the species-specific reverse primers 18SFrontR-LT, 18SFrontR-LS, and 18SFrontR-AG used with the conserved forward primer, 18SFrontF, were able to discriminate the parasitoid species from one another and from the host aphid in a PCR (Fig. 3). When host aphid DNA was amplified with the conserved forward and the parasitoid species-specific reverse primers, no products were formed. The same

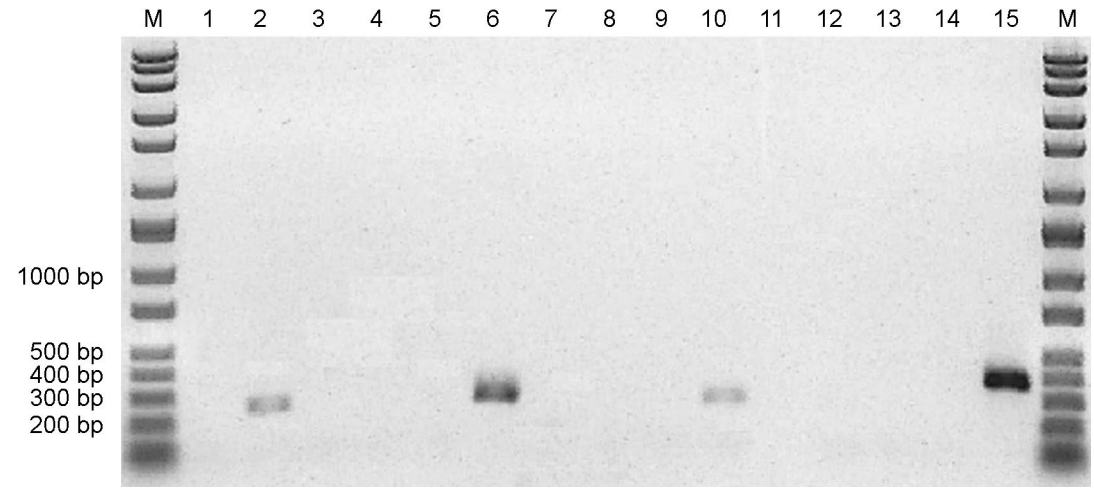

Fig. 3. PCR products from 18S rDNAs amplified using 18SFrontF and species-specific reverse primers to differentiate parasitoids from each other and the host aphid. Lane M, wide range DNA marker (Sigma-Aldrich). Lane 1, negative control. Lanes 2–4, parasitoid DNAs (*A. gossypii*, *L. testaceipes*, and *L. scutellaris*, respectively) amplified with 18SFrontF and 18SFrontR-AG primers. Lanes 5–7, parasitoid DNAs amplified with 18SFrontF and 18SFrontR-LT primers. Lanes 8–10, parasitoid DNAs amplified with 18SFrontF and 18SFrontR-LS primers. Lane 11, blank. Lanes 12–14, *T. citricida* DNA amplified with 18SFrontF and 18SFrontR-AG, -LT, and -LS primers, respectively. Lane 15, positive control for *T. citricida* DNA amplified with 18SFrontF and 18SFrontI1R primers.

host template was amplified with both conserved forward and reverse primers in a positive control, demonstrating the presence of host DNA in the reaction mixture (Fig. 3). When mixtures of host aphid and parasitoid DNAs were amplified with the conserved forward primer and each species-specific reverse primer, the results (Fig. 4) were similar to those observed in Fig. 3. The 18SFrontF and 18SFrontR-AG primer pair amplified a 255-bp fragment, the 18SFrontF and 18SFrontR-LT primer pair amplified a 265-bp fragment, and the 18SFrontF and 18SFrontR-LS primer pair amplified a 269-bp fragment as expected. The 18SFrontF and 18SFrontI1R primer pair amplified a 381-bp fragment in a positive control containing only host aphid DNA. The presence of host DNA in the reaction mixture did not affect the performance of each species-specific reverse primer (Fig. 4).

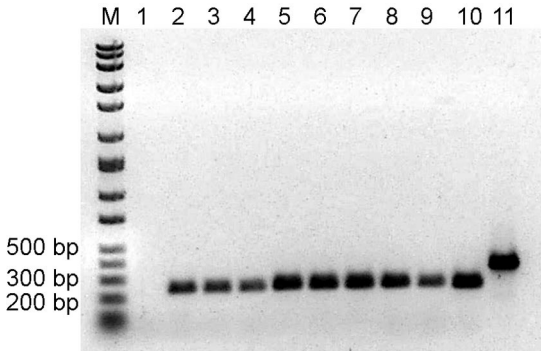

Fig. 4. PCR products from DNA mixtures of brown citrus aphid and parasitoids by using 18SFrontF and species-specific reverse primers. Lane M, wide range DNA marker (Sigma-Aldrich). Lane 1, negative control. Lanes 2–4, DNA mixtures of *T. citricida* and *A. gossypii* amplified with 18SFrontF and 18SFrontR-AG primers. Lanes 5–7, DNA mixtures of *T. citricida* and *L. testaceipes* amplified with 18SFrontF and 18SFrontR-LT primers. Lanes 8–10, DNA mixtures from *T. citricida* and *L. scutellaris* amplified with 18SFrontF and 18SFrontR-LS primers. Lane 11, positive control for *T. citricida* DNA amplified with 18SFrontF and 18SFI1R primers.

This approach was similar to that of Zhu and Greenstone (1999) and Zhu and Williams (2002) to detect hymenopteran parasitoids within the host insects. In those experiments, rDNA sequences for the ITS2 region were used to design primers that discriminated parasitoids and host DNAs. It is important to consider that intragenomic and intraindividual variation occurs among some rDNA sequences as noted by Onyabe and Conn (1999) and Alvarez and Hoy (2002). This variation can influence the results of phylogenetic analyses and discriminating experiments based on gene sequences that may be too variable for the intended purpose. It is suggested that the most conserved gene and approach available be used in such experiments to achieve purpose without sacrificing reliability of the results. The extremely conserved 18S rRNA gene was used in this experiment to reduce the probability of encountering intraspecific and intraindividual variation that has been observed among other less conserved insect rRNA genes.

Temporal Effects on Parasitoid Detection. There was a significant effect of time on the detection of *L. testaceipes* within parasitized brown citrus aphids by PCR ($F = 105.6$; df = 2, 17; $P = 0.0001$). The relationship between percentage of parasitoids detected and time from parasitoid oviposition within the aphid was described by the quadratic equation y = 8.70

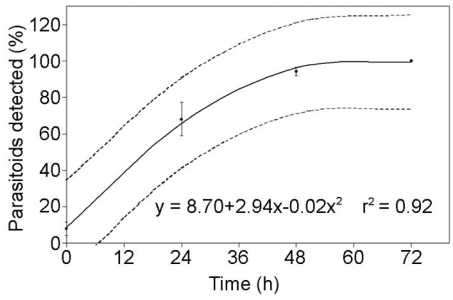

Fig. 5. Relationship between percent parasitism by *L. testaceipes* detected by PCR and time from parasitoid oviposition within the host aphid, *T. citricida.* A quadratic model was fitted to the data to develop the prediction equation. Vertical lines denote standard errors of the means and dashed lines denote 95% CL for the prediction equation.

$(\pm 4.88) + 2.94\ (\pm 0.33)x - 0.02\ (\pm 0.004)x^2$; $r^2 = 0.92$ (Fig. 5). The Durbin–Watson d statistic was 1.916, indicating that the errors were not autocorrelated due to the time-series arrangement of the data. Detection of parasitoid DNA within the host aphid was possible in 8.0 ± 3.7% of samples within 2 h after oviposition, in 66.0 ± 9.2% of samples after 24 h, in 94.0 ± 2.4% of samples after 48 h, and in 100% of samples after 72 h.

Detection of parasitoid DNA by PCR from a mixture of parasitoid and host DNA may be affected by several factors. The ratio of parasitoid to aphid DNA would vary among samples due to differences in the physiological stages and fitness of both the parasitoid and aphid. The success of the procedure depends on the detection limit of the PCR and the amount of parasitoid DNA present in the sample. The detection rate observed in this experiment was lower within 2 and 24 h after oviposition probably because the concentration of the parasitoid 18SrDNA gene present in the sample was near or below the detection limit of the PCR. It has been reported that the egg of *L. testaceipes* hatches ≈48 h after it is oviposited in the host aphid (Knutson et al. 1993). In this experiment, parasitoid DNA was detected in 94 and 100% of samples after 48 and 72 h, respectively. Hatching of the parasitoid egg apparently improved the ability to detect parasitoid DNA in the mixture. Because the egg of the parasitoid is minute, the manual force used during grinding of samples may also contribute to differences in the ratio of parasitoid to aphid DNA. The grinding procedure might be improved by extending the period of grinding and using liquid nitrogen. Additionally, the detection sensitivity of PCR may be improved by increasing the number of thermocycles beyond that used in this experiment.

The PCR approach described in this experiment is faster and more accurate than rearing or dissection methods previously available to monitor parasitism within aphid populations. Developmental time was 10–14 d for *L. testaceipes* and 14–18 d for *A. gossypii* on a related host species, *Toxoptera aurantii* (Boyer de Fonscolombe), with mortality rates of 10–20% at temperatures normally experienced in Florida (Tang and Yokomi 1995). Because identification of the larval stages of parasitic Hymenoptera is difficult even for experienced taxonomists, the advantages of early detection and accuracy of identification provided by PCR are evident. Monitoring the establishment of imported parasitoids and their competition with native parasitoids is facilitated by this approach. The procedure is relatively inexpensive and provides an alternative to traditional methods for examining parasitoid activity against brown citrus aphids.

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